User Manual

ValidPrime™

Control for Genomic Background

Human and *Mouse*

Probe protocol

Version 1.1— September 2012

For use in quantitative real-time PCR

tataabiocenter

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- (HL)-dsDNase
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Background

For accurate gene expression analysis, the measured C_q shall reflect the amount of gene transcript present in the sample. This requires that the assay is specific and selective for the targeted cDNA and contributions to the signal from primer dimers, pseudogenes and genomic DNA are negligible. To test for primer dimer formation qPCR is performed in absence of template (no template control, NTC) and to test for genomic DNA (gDNA) background reverse transcription (RT) is performed in the absence of reverse transcriptase (RT(-)). The NTC is sample independent and is performed only once to validate assay performance, while the amount of gDNA may vary and RT(-) controls are typically measured on all samples. These controls add substantially to the cost of a qPCR study.

ValidPrime™ is an assay to test for the presence of gDNA in test samples and when combined with a gDNA control sample, replaces all RT(-) controls. ValidPrime™ is highly optimized and specific to a non-transcribed locus of gDNA that is present in exactly one copy per haploid normal genome. Therefore, ValidPrime™ measures the number of genomic copies present in a sample and can be used for normalization of samples to cell copy number, as endogenous control for CNV applications, and as control for gDNA background in RTqPCR. The ValidPrime™ kit also contains a gDNA standard that can be used to test the sensitivity of RTqPCR assays for gDNA background.

In expression profiling experiment the ValidPrime™ assay is added to the list of assays and the gDNA control is added to the list of samples. From the combined measurements with the ValidPrime™ assay and the gene of interest (GOI) assays on all samples and on the gDNA control the genomic background contribution to all RTqPCR measurements can be assessed. ValidPrime™ replaces the need to perform RT(-) controls for all reactions and makes RTqPCR profiling easier and substantially cheaper. In an expression profiling experiment based on *m* samples and *n* assays, traditional set up requires *m* RT(-) reactions plus *m* x *n* qPCR controls, while using ValidPrime™ only *m* + *n* + 1 controls are needed (Table 1).

No. of controls		Assays (*n*)									
		1		10		24		48		96	
Samples (*m*)	1	2	3	11	12	25	26	49	50	97	98
	10	20	12	110	21	250	35	490	59	970	107
	24	48	26	264	35	600	49	1176	73	2328	121
	48	96	50	528	59	1200	73	2352	97	4656	145
	96	192	98	1056	107	2400	121	4704	145	9312	193

Traditional RT(-) strategy | ValidPrime™

Table 1: Total number of RT and qPCR controls needed to check for gDNA background using traditional RT(-) approach and ValidPrime™. In an expression profiling experiment based on *m* samples and *n* assays, traditional set up requires *m* RT(-) reactions plus *m* x *n* qPCR controls, while using ValidPrime™ only *m* + *n* + 1 controls are needed.

Traditional approach based on RT(-) controls

Presence of genomic background in RTqPCR expression profiling is conventionally assessed by running an RT(-) control for each sample that is analyzed by qPCR for all the GOI's. Any signal observed in these RT(-)qPCR's is due to presence of contaminating DNA that is amplified by the qPCR assay designed for GOI. A common criterion to accept the measured C_q as not being confounded by gDNA contamination is $Cq^{GOI}_{RT(-)} - Cq^{GOI}_{RT(+)} > 5$. The estimated GOI concentration is then accurate to at least 96.9 % (Figure 1).

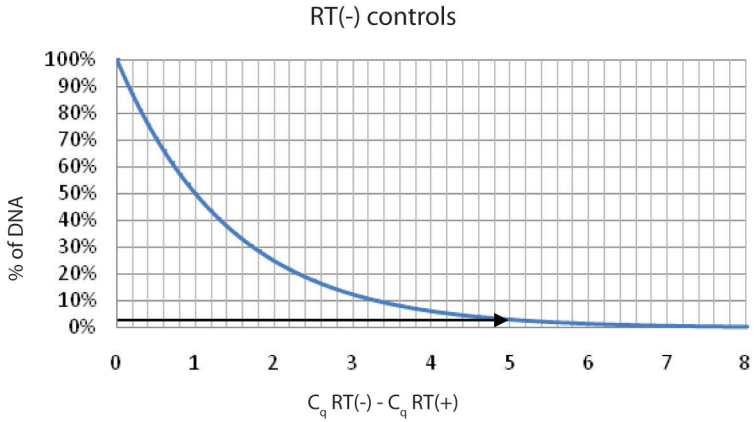


Figure 1: Correlation of ΔC_q RT(-)-RT(+) with percentage of gDNA in unknown sample

If $Cq^{GOI}_{RT(-)} - Cq^{GOI}_{RT(+)} < 5$ the measured $Cq^{GOI}_{RT(-)}$ is confounded. It can be corrected to reflect the RNA concentration using eq. 1 (Laurell *et al.*, 2011):

$$Cq^{GOI}_{RNA} = -\log_2\left(2^{-Cq^{GOI}_{RT(+)}} - 2^{-Cq^{GOI}_{RT(-)}}\right)$$

Equation 1

$Cq^{GOI}_{RT(+)}$ and $Cq^{GOI}_{RT(-)}$ are the qPCR C_q values measured for the RT(+) and RT(-) reactions, and Cq^{GOI}_{RNA} is the Cq value that would have been obtained for the RT(+) reaction in absence of gDNA contaminations. From Cq^{GOI}_{RNA} the correct transcript amount can be calculated.

ValidPrime™

Using ValidPrime™ the same test for gDNA contamination can be performed and, if needed, the same correction for background can be made, with a much smaller number of reactions. The sensitivities of the GOI qPCR assays for gDNA (Cq^{GOI}_{gDNA}) are tested relative to the ValidPrime™ assay ($Cq^{ValidPrime}_{gDNA}$) on the provided gDNA standard. Well performing GOI assays that have been properly designed to exclusively target mRNA by, for example, having intron spanning primers shall not amplify the gDNA standard, while GOI assays that amplify sequences present in multiple copies in the gDNA will have even lower C_q values than the ValidPrime™ assay. All samples are then analyzed also with the ValidPrime™ assay ($Cq^{ValidPrime}_{Sample}$). The measurement setup is shown in Table 2.

Original data	GOI 1	GOI 2	GOI 3	GOI 4	ValidPrime™
Sample 1	20.1	31.1	22.1	28.2	32.5
Sample 2	20.5	31.2	22.5	28.9	33.2
Sample 3	21.0	31.1	22.9	30.2	32.3
Sample 4	23.1	31.8	22.5	32.3	34.2
Sample 5	23.5	30.8	22.8	32.0	33.1
gDNA standard	***25.8***	***26.9***	***26.7***	***26.0***	***27.0***

Table 2: Experimental setup based on five samples assayed for four GOI's and ValidPrime™ including also the gDNA standard. $Cq^{GOI}_{RT(+)}$ is shown in black, Cq^{GOI}_{gDNA} in blue, $Cq^{ValidPrime}_{Sample}$ in red, $Cq^{ValidPrime}_{gDNA}$ in ***orange***.

From the measured $Cq^{ValidPrime}_{Sample}$,$Cq^{ValidPrime}_{gDNA}$ and Cq^{GOI}_{gDNA} expected C_q for RT(-) controls, $Cq^{GOI}_{RT(-)}$ are calculated with Equation 2 and, as before, Equation 1 is used to correct for the gDNA background (Table 3).

$$Cq^{GOI}_{RT(-)} = Cq^{GOI}_{gDNA} + \left(Cq^{ValidPrime}_{Sample} - Cq^{ValidPrime}_{gDNA}\right)$$

Equation 2

ValidPrime™	Gene 1			Gene 2			Gene 3			Gene 4		
	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}
Sample 1	20.1	31.3	20.10	31.1	32.4	31.85	22.1	32.2	22.10	28.2	31.5	28.35
Sample 2	20.5	32.0	20.50	31.2	33.1	31.65	22.5	32.9	22.50	28.9	32.2	29.05
Sample 3	21.0	31.1	21.00	31.1	32.2	32.01	22.9	32.0	22.90	30.2	31.3	31.11
Sample 4	23.1	33.0	23.10	31.8	34.1	32.13	22.5	33.9	22.50	32.3	33.2	33.41
Sample 5	23.5	31.9	23.50	30.8	33.0	31.15	22.8	32.8	22.80	32.0	32.1	35.90

Table 3: Measured $Cq^{GOI}_{RT(+)}$ calculated $Cq^{GOI}_{RT(-)}$ using Equation 2, and calculated Cq^{GOI}_{RNA} using Equation 1.

Contents

• A 6 months complimentary license for GenEx Enterprise - the easiest way to correct RTqPCR data for gDNA background. GenEx is market leading software for qPCR experimental design and data processing. Read more on page 11. 1 GenEx license per customer, for first time users only.

• Reference standard gDNA 50 µl or 100 µl, C=200 ng/µl.
We recommend using 10 ng per qPCR.

• ValidPrime™ assay primers for:
250 rxns* (250 µl of primer mix, C=10 µM per primer)
or 1000 rxns* (1000 µl of primer mix, C=10 µM per primer)

• ValidPrime™ assay probe for:
250 rxns* (125 µl of solution, C=10 µM)
or 1000 rxns* (500 µl of solution, C=10 µM)

* rxns = qPCR reactions in 25 µl, concentration = 400 nM per primer, 200 nM probe

The ValidPrime™ assay amplifies a gDNA sequence that is present in exactly one copy per haploid genome in a normal cell. The sequence has no transcriptional activity and is not present in pure cDNA preparations. The assay has very high PCR efficiency (E > 90% in tested commercial master mixes) and produces negligible amount of primer dimer products. Limit of detection (LOD) is estimated to 4 copies of DNA (0.01 ng of DNA), limit of quantification (LOQ) is estimated to 32 copies of DNA (0.08 ng of DNA). Probe is using FAM reporter and BHQ1 quencher.

Storage

The ValidPrime™ kit can be stored for 1 month at +4°C. For long term storage -20°C is recommended. Repeated freeze-thaw cycles should be avoided. Vortex thoroughly and spin down before use.

Additionally required materials and devices

• qPCR instrumentation

The ValidPrime™ kit has been validated on:
Roche LightCycler 480, Biorad CFX, Stratagene MxPro, Rotorgene, ABI 7500 Fast and is expected to perform excellent on related instruments. The ValidPrime™ probe signal shall be measured on the instrument's FAM channel.

• Master mix or master mix components

The ValidPrime™ kit has been validated in a large number of master mixes using the conditions recommended by the manufacturers, including:

Master mix	Final concentrations*	Annealing temperature
Applied Biosystems TaqMan® Gene expression Master Mix	800 nM primer, 200 nM probe	= 61°C
Applied Biosystems TaqMan® Universal PCR Master Mix	800 nM primer, 200 nM probe	= 59°C
Applied Biosystems TaqMan® Genotyping Master Mix	800 nM primer, 200 nM probe	= 59°C
Finnzymes DyNAmo™ Flash Probe qPCR Kit	500 nM primer, 250 nM probe	≈ 60°C
Finnzymes DyNAmo™ ColorFlash Probe qPCR Kit	500 nM primer, 250 nM probe	≈ 60°C
KAPA™ PROBE FAST qPCR Kit	400 nM primer, 200 nM probe	≈ 60°C
Qiagen QuantiTect Probe PCR Kit	400 nM primer, 200 nM probe	≈ 60°C
Roche LC480 Probes master	500 nM primer, 200 nM probe	≈ 60°C

* Concentration per primer and probe in qPCR

Table 4: Recommended primer and probe concentrations, and annealing temperature in selected commercial master mixes

• Pipettes and tips (available from www.tataa.com)

• Vortex and centrifuge

• Sample RNA/DNA

• Optionally DNase

With ValidPrime™ measured $Cq^{GOI}_{RT(+)}$ can be corrected for up to 50% gDNA background. If gDNA background is high it is recommended to reduce it by treating

the cDNA with double strand specific DNase that will remove specifically the gDNA and will not degrade the cDNA. We recommend heat labile dsDNase (HL-dsDNase), which is efficiently heat inactivated and does not digest the PCR product either. HL-dsDNase is available from www.tataa.com.

• Optionally reference cDNA
Newly designed assays can be validated on cDNA libraries. Several cDNA libraries are available from www.tataa.com.

Amplification protocol

Use the recommended amplification protocol for your master mix. Optimal annealing temperature of the ValidPrime™ assay is about 60°C in most master mixes, see table 4 for the optimum conditions in selected master mixes. Either 2- or 3-step amplification protocol can be used.

Pipetting protocol

Prepare a master mix for each assay using the protocol from the manufacturer, including the recommended concentrations of primers and probe . Prepare for at least one extra reaction so you do not run out of master mix during the pipetting. An NTC is recommended to test for contamination of reagents. We recommend 10 ng of gDNA per 10 µl qPCR, which on most qPCR instruments should produce a C_q for the ValidPrime™ assay in the range 25-30 cycles.

GenEx

Easiest is to use GenEx for correction of RTqPCR data for gDNA background and for general qPCR data processing. GenEx is market leading software for qPCR experimental design and data processing, and is supported by all leading qPCR instrument manufacturers. It offers user friendly optimized workflow for qPCR data pre-processing and analysis. Pre-processing includes interplate calibration, efficiency correction, various normalization options, handling of technical replicates and missing data, normalization with paired samples and correction for gDNA contamination using ValidPrime™. Analyses include absolute quantification, relative quantification and expression profiling. Tutorials are available on: www.multid.se/tutorials.php and free support is offered on www.qpcrforum.com.

A 6 months complimentary license for GenEx Enterprise is included for first time users with the ValidPrime™ kit. To get started, send an email to info@multid.se and state your order number for your ValidPrime kit together with your customer details. You will receive a key to activate your free license that can be downloaded from www.multid.se. To purchase additional GenEx licenses or for qPCR data analysis services, contact us on order@tataa.com.

Troubleshooting

• I do not get any amplification/signal?
The instrument may not have been programmed correctly or there may be a problem with the master mix. Establish if the problem is in the detection or the amplification by running the samples on a gel. Run a new test using the gDNA control with the ValidPrime™ assay provided.

• My negative controls are amplified?
Your reagents are probably contaminated.

• My samples have same/higher C_q-value than my NTC?
You have used too little cDNA. Add more cDNA and try again. The cDNA may also be of poor quality. Check the quality of the RNA before performing cDNA synthesis.

• My replicates are not tight?
With good quality cDNA and good pipetting technique, very high reproducibility is expected. Low amounts of cDNA can lead to higher variation. Also, low quality cDNA can lead to differences between replicates. Check the accuracy and reproducibility of your pipettes. It is also possible the qPCR instrument is malperforming.

• I get positive ValidPrime™ signal even after DNAse treatment?
Often DNAse treatment does not remove all DNA and qPCR will amplify a single molecule. Usually solution based DNase treatment is more efficient than column based DNase treatment. You may also try the HL-dsDNase from TATAA (www.tataa.com), which has superior performance to competing dsDNases. Usually DNase treatment reduces the gDNA background enough to be accounted for by ValidPrime™ and GenEx correction.

References

Henrik Laurell, Jason Iacovoni, David Svec, Anne Abot, Jean-José Maoret, Jean-François Arnal, Mikael Kubista. Correcting RTqPCR data for genomic DNA background. Nucleic Acids Res. 2012.

Mikael Kubista, Vendula Rusnakova, David Svec, Björn Sjögreen and Ales Tichopad. GenEx - Data Analysis Software. In qPCR in Applied Microbiology. Editor: Martin Filion. Horizon Press, 2012.

Reorder information

The ValidPrime™ kit can be ordered from TATAA by mail at order@tataa.com, from our TATAA webshop on www.tataa.com or from the local TATAA distributor in your country.

Contact

For more information about ValidPrime™, contact us at info@tataa.com.

License information

PCR is covered by several patents owned by Hoffman-La Roche Inc., and Hoffman-LaRoche, Ltd. Purchase of the ValidPrimeTM kit does not include or provide a license with respect to any PCR related patents owned by Hoffman-La Roche or others. TATAA Biocenter does not encourage or support the unauthorised or unlicensed use of the PCR process.

Other products from TATAA

(HL-)dsDNase

New generation DNase that is specific to double stranded DNA and can be efficiently inactivated by heating at 55 °C. It can be added to your RT reaction to efficiently remove any gDNA, without degrading single-stranded cDNA. It is completely inactivated by the PCR and does not degrade the double stranded PCR product.

GenEx software

Market leading software for qPCR analysis. GenEx provides the appropriate tools to analyze qPCR gene expression data and to extract biologically relevant information from the measurements.

Reference Gene Panel - Human or Mouse

The panel contains primer sets for 12 commonly used human or mouse reference genes. A perfect product for finding the most optimal reference gene for your samples. A one year license of GenEx Standard software with GeNorm and Normfinder is also included in the kit.

VisiBlue™ qPCR mix colorant

The VisiBlue mastermix colorant enables you to color your favourite qPCR mastermix to easily visualize where the reagent is loaded to your plates and tubes. VisiBlue is very easy to use by simple addition to your favorite master mix.

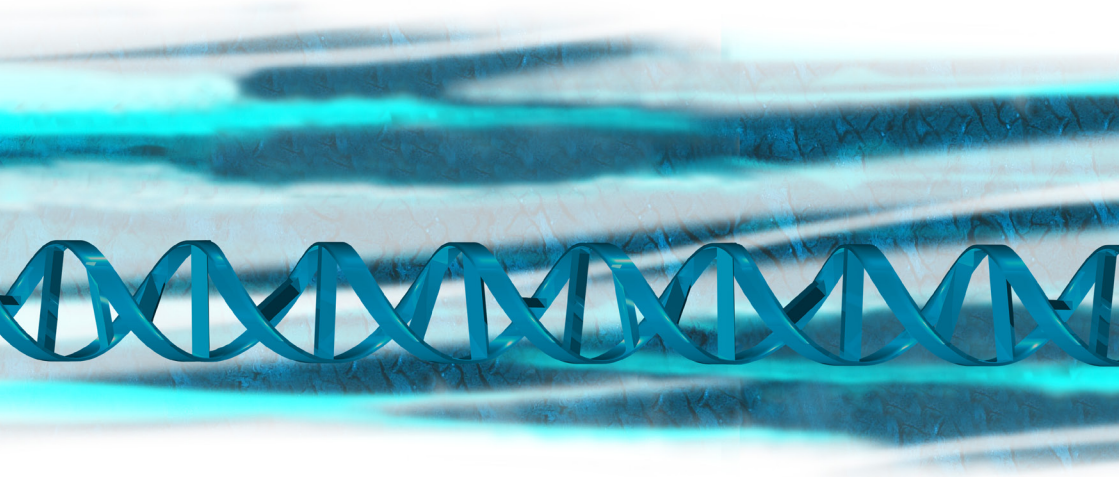
CelluLyser™ - for rapid and easy lysis and cDNA synthesis

The CelluLyser™ Lysis and cDNA Synthesis Kit enables you to generate cDNA from small samples with minimal losses and hands-on time. It is particularly useful for single cell analysis. By using CelluLyser™, the entire workflow from cell lysis to RT and qPCR can be performed without washing steps, thus eliminating material loss.

TATAA Interplate Calibrator - Variation Compensation

For practical reasons many qPCR studies involve the use of samples that are processed in more than a single batch or in which the sample set is extended over time. Even over a short time period, variation between qPCR processing runs is observed due to different baseline subtractions and threshold settings. The TATAA Interplate Calibrator (IPC) is used to compensate for the variation between qPCR runs.

Express your genius



TATAA Biocenter, with offices in Gothenburg, San Francisco and Prague is the leading provider of real-time PCR services and the prime organizer of real-time PCR workshops globally. TATAA Biocenter conducts commissioned research and training within the field of molecular diagnostics and gene expression analysis, along with developing real-time PCR expression panels. TATAA Biocenter has great experience and expertise in high resolution gene expression profiling, pathogen detection, and small sample/single cell analysis.

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